

University of Groningen

Mutational Analysis of the Role of the Glucansucrase Gtf180-Delta N Active Site Residues in Product and Linkage Specificity with Lactose as Acceptor Substrate

Pham, Hien; Pijning, Tjaard; Dijkhuizen, Lubbert; van Leeuwen, Sander Sebastiaan

Published in:
Journal of Agricultural and Food Chemistry

DOI:
[10.1021/acs.jafc.8b04486](https://doi.org/10.1021/acs.jafc.8b04486)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Pham, H., Pijning, T., Dijkhuizen, L., & van Leeuwen, S. S. (2018). Mutational Analysis of the Role of the Glucansucrase Gtf180-Delta N Active Site Residues in Product and Linkage Specificity with Lactose as Acceptor Substrate. *Journal of Agricultural and Food Chemistry*, 66(47), 12544-12554. [acs.jafc.8b04486]. <https://doi.org/10.1021/acs.jafc.8b04486>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.



Mutational Analysis of the Role of the Glucansucrase Gtf180-ΔN Active Site Residues in Product and Linkage Specificity with Lactose as Acceptor Substrate

Hien Pham,[†] Tjaard Pijning,[‡] Lubbert Dijkhuizen,^{*,†,§} and Sander S. van Leeuwen^{†,||}

[†]Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

[‡]Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

S Supporting Information

ABSTRACT: Glucansucrase Gtf180-ΔN from *Lactobacillus reuteri* uses lactose as acceptor substrate to synthesize five glucosylated lactose molecules (F1–F5) with a degree of polymerization (DP) of 3–4 (GL34) and with (α1→2)/(α1→3)/(α1→4) glycosidic linkages. Q1140/W1065/N1029 mutations significantly changed the GL34 product ratios. Q1140 mutations clearly decreased F3 3'-glc-lac with an (α1→3) linkage and increased F4 4',2-glc-lac with (α1→4)/(α1→2) linkages. Formation of F2 2-glc-lac with an (α1→2) linkage and F4 was negatively affected in most W1065 and N1029 mutants, respectively. Mutant N1029G synthesized four new products with additional (α1→3)-linked glucosyl moieties (2xDP4 and 2xDP5). Sucrose/lactose strongly reduced Gtf180-ΔN hydrolytic activity and increased transferase activity of Gtf180-ΔN and mutant N1029G, in comparison to activity with sucrose alone. N1029/W1065/Q1140 thus are key determinants of Gtf180-ΔN linkage and product specificity in the acceptor reaction with lactose. Mutagenesis of key residues in Gtf180-ΔN may allow synthesis of tailor-made mixtures of novel lactose-derived oligosaccharides with potential applications as prebiotic compounds in food/feed and in pharmacy/medicine.

KEYWORDS: glucansucrase, lactose, oligosaccharides, protein engineering, prebiotics, transglycosylation, *Lactobacillus reuteri*

INTRODUCTION

Glucansucrases (Gtfs) are extracellular transglycosidases found in lactic acid bacteria and belong to glycoside hydrolase family 70 (GH70).^{1–3} These enzymes synthesize α-glucan polymers from sucrose as acceptor and donor substrate, in a semi-processive manner.⁴ Glucansucrases have also been shown to efficiently catalyze transfer of glucose moieties from sucrose as the donor substrate to numerous hydroxyl group containing molecules, including maltose, isomaltose, catechol, primary alcohols (C₄, C₆, and C₈), and steviol-based compounds.^{5–10} In case of small sugar acceptor molecules, low-molecular-mass oligosaccharides are synthesized, differing in linkage type, size, branching degree, and physicochemical properties.^{1,3,10,11} These products are attracting strong interest for industrial applications as food or feed ingredients and in pharmacy and medicine.^{12–15}

GH70 glucansucrases belong to the α-amylase superfamily, together with GH13 and GH77 enzymes,^{16,17} but they are much larger (~1600–1800 amino acid residues). An N-terminal domain of variable length and a C-terminal putative glucan-binding domain flank the central catalytic domain in these glucansucrase enzymes.¹ GH70 enzymes follow a double-displacement reaction mechanism and possess three catalytic residues, D102S (nucleophile), E1063 (acid/base), and D1136 (transition state stabilizing residue) (Gtf180-ΔN numbering). The reaction starts with cleavage of the (α1→2) bond of sucrose, yielding a covalent glucosyl-enzyme intermediate. This is followed by binding of the acceptor substrate and transfer of

the covalently bound glucosyl residue to the acceptor molecule, forming a new glycosidic linkage.¹⁸ The α-anomeric configuration of the donor is conserved in the product.^{19,20} The different glucansucrases characterized produce α-glucans with various types of linkages, depending on the orientation of the acceptor glucan toward the covalent glucosyl-enzyme intermediate. The glucansucrase linkage specificity, therefore, is determined by residues forming the acceptor-binding subsites.²¹ The glucansucrase binding subsites for acceptor substrates are relatively open and involve several conserved sequence regions. Residues forming subsites +1 and +2 are crucial for acceptor recognition and orientation; their mutation resulted in altered ratios of glycosidic linkages in the synthesized α-glucans.^{22–24}

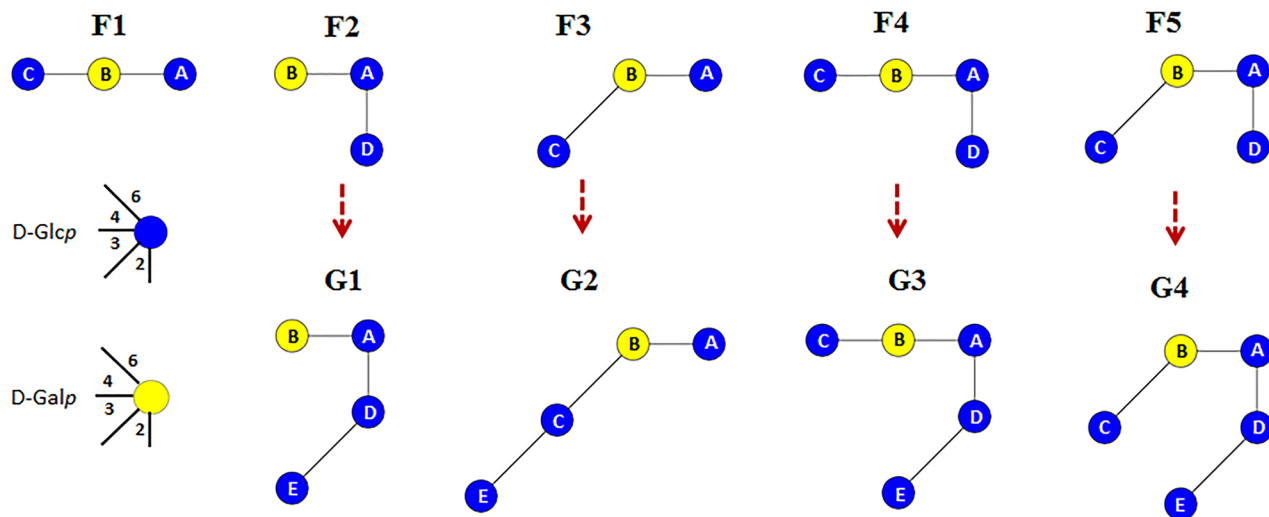
The Gtf180-ΔN glucansucrase from *Lactobacillus reuteri* 180 synthesizes an α-glucan with (α1→6) and (α1→3) glycosidic linkages.²⁵ Its three-dimensional structure has been elucidated,¹⁸ also with bound donor substrate (sucrose; PDB code 3HZ3) and with acceptor substrate (maltose; PDB code 3KLL), identifying amino acid residues in the donor and acceptor substrate binding subsites.¹⁸ The crystal structure of Gtf180-ΔN revealed five protein domains (A, B, C, IV, and V); the active site is situated at the interface of domains A and B

Received: August 29, 2018

Revised: November 5, 2018

Accepted: November 6, 2018

Published: November 6, 2018

Scheme 1. Structures of F1–F5 and G1–G4^a

^aRed arrows reflect possible elongation of the corresponding compounds from the mixture GL34, F2–F5, to form G1–G4.

with a pocketlike cavity;²⁶ residues Q1140, N1411, and D1458 flanking subsite –1 prevent the presence of further donor subsites.¹⁸ The crystal structure of Gtf180-ΔN in a complex with maltose revealed this acceptor substrate bound in subsites +1 and +2. At subsite +1, the highly conserved residue N1029 from domain A provides direct and indirect hydrogen bonds to the C3 and C4 hydroxyl groups at the nonreducing end of maltose.¹⁸ Residue W1065 has a hydrophobic stacking interaction with both the +1 and +2 glucosyl units of maltose, while the complex of Gtf180-ΔN D1025N with sucrose revealed a direct hydrogen bond of W1065 with the C1 hydroxyl group of the fructosyl moiety of sucrose.²¹

Our previous study showed that Gtf180-ΔN successfully catalyzes transglycosylation reactions from sucrose with lactose as the acceptor substrate.²⁷ Multiple glucose moieties were transferred to lactose to produce various glucosylated lactose derivatives. The five compounds F1–F5 of DP3–DP4 (GL34 mixture) were structurally characterized revealing (α 1→2), (α 1→3), and (α 1→4) linkages (Scheme 1).²⁷ Interestingly, Gtf180-ΔN introduced an (α 1→2)-linked Glc moiety at the reducing glucosyl unit of lactose. Such a glycosidic linkage specificity had not been reported for this enzyme before. It remained unknown how the (α 1→6)/(α 1→3) linkage specificity of Gtf180-ΔN was altered to (α 1→2)/(α 1→3)/(α 1→4) in the presence of lactose as an acceptor substrate. The branching sucrase enzyme Dsr-E from *Leuconostoc mesenteroides* NRRL B-1299 is one of the rare enzymes in the GH70 family that is able to introduce (α 1→2)-branched linkages onto dextran backbones.^{28,29} Aiming to understand how the acceptor substrate lactose binds in the Gtf180-ΔN active site, and which amino acids are essential in binding lactose, we carried out docking experiments with lactose in a glucosyl-enzyme intermediate, using the crystal structure of *L. reuteri* 180 Gtf180-ΔN.¹⁸ Residues N1029, W1065, and Q1140 were found to be in close proximity of the acceptor substrate and may therefore be involved in the orientation of lactose in the acceptor subsite and influence the linkage type preference. To study this in more detail, mutants at these positions were biochemically characterized and evaluated for their lactose-derived product spectra and linkage specificity. The results show that mutagenesis of key residues in Gtf180-ΔN may

allow synthesis of tailor-made mixtures of lactose-derived oligosaccharides with various linkage types. Such mixtures of lactose-derived oligosaccharides may have potential applications as prebiotic compounds in food and feed and in pharmacy and medicine.

MATERIALS AND METHODS

Recombinant Gtf180-ΔN (Mutants). Mutation, expression, and purification of the Gtf180-ΔN mutant enzymes used for this study has been described in detail by Meng et al.^{30,31}

Glucansucrase Activity and Kinetic Analysis. Glucansucrase activity was quantified by measuring released glucose and fructose in the reaction with 100 mM sucrose at 37 °C in 25 mM sodium acetate buffer (pH 4.7) with 1 mM CaCl₂, as described previously.³² Samples of 25 μ L were taken at regular intervals of 1 min, and glucosylation reactions were stopped immediately by addition of 5 μ L of 1 M NaOH. One glucansucrase activity unit (U) is defined as the amount of enzyme releasing 1 μ mol of monosaccharide from sucrose per minute. Release of glucose and fructose corresponds to hydrolysis activity and total activity, respectively, and the transglycosylation activity was calculated as the difference. Kinetic parameters (V_{\max} and K_m) were determined using 10 different sucrose concentrations (ranging from 0.1 to 200 mM) with 200 mM lactose as acceptor substrate and calculated by nonlinear regression of the Michaelis–Menten equation with SigmaPlot. The effect of lactose concentration on initial activities of Gtf180-ΔN and mutants derived were determined using eight different lactose concentrations (ranging from 0.1 to 150 mM) in the reactions with 150 mM sucrose at 37 °C in 25 mM sodium acetate buffer (pH 4.7) containing 1 mM CaCl₂.

Docking Experiments. A model of the covalent glucosyl-enzyme intermediate of Gtf180-ΔN from *L. reuteri* strain 180 was used to dock lactose as an acceptor substrate, using the Vina-Carb of AutoDock Vina.^{18,33,34} Amino acid side chains were kept rigid. Seventy-seven poses were obtained, and these were evaluated in PyMOL (The PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC) on the basis of their binding energy, proximity, and orientation of the hydroxyl group with respect to the C1 atom of the covalent glucosyl moiety. The result was a collection of 34 poses in which the distance of the relevant lactose hydroxyl group to the C1 atom of the glucosyl-enzyme intermediate ranged between 3.0 and 4.6 Å; these poses were considered productive.

Mutant Enzyme Screening. Wild type and mutant Gtf180-ΔN enzymes (1 U mL^{−1}, total activity, with sucrose) were incubated with 0.5 M sucrose and 0.3 M lactose. Control incubations contained only 0.5 M sucrose with 1 U mL^{−1} of these enzymes. All reactions were

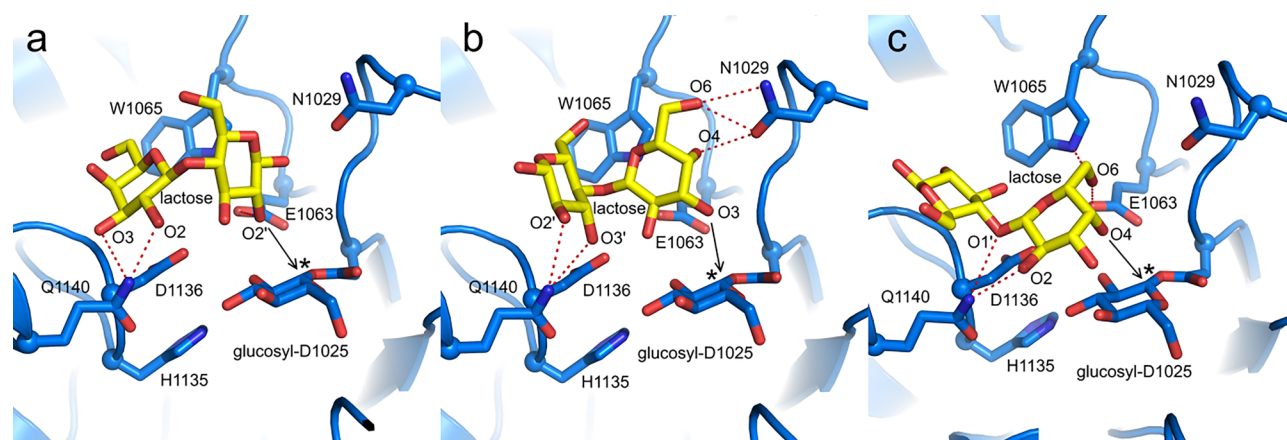


Figure 1. Views of the acceptor substrate lactose (yellow carbon atoms) docked in a glucosyl-enzyme intermediate constructed using the crystal structure of *L. reuteri* 180 Gtf180-ΔN (PDB 3KLK¹⁸). Three different poses are shown representing (a) (α1→2) transglycosylation at the reducing end, (b) (α1→3) transglycosylation at the nonreducing end, and (c) (α1→4) transglycosylation at the nonreducing end. Hydrogen bonds between amino acid residues and lactose are shown as red dotted lines; the arrow indicates the relevant hydroxyl group of lactose to attack the C1 atom of the glucosyl-enzyme intermediate (indicated with an asterisk).

performed at 37 °C in 25 mM sodium acetate buffer (pH 4.7) with 1 mM CaCl₂ for 24 h. The resulting reaction mixtures were subsequently subjected to HPAEC-PAD profiling for analysis.

Larger Scale Production and Isolation of Oligosaccharides.

Larger scale reactions of 0.5 M sucrose and 0.3 M lactose with selected Gtf180-ΔN mutants were carried out in a volume of 100 mL with 1 U mL⁻¹ (total activity) of enzyme for 24 h. Two volumes of cold ethanol 20% were added to the reaction mixtures and stored at 4 °C overnight to precipitate glucan polysaccharides. Full precipitation was promoted by centrifugation at 10000g for 10 min, and the supernatant was applied to a rotatory vacuum evaporator to remove ethanol. The aqueous fraction was absorbed onto a CarboGraph SPE column (Alltech, Breda, The Netherlands) using acetonitrile/water 1/3 as eluent, followed by evaporation of acetonitrile under an N₂ stream before being freeze-dried. Oligosaccharide mixtures were fractionated by HPAEC-PAD.

HPAEC-PAD Analysis. Analytical scale HPAEC-PAD analyses were performed on a Dionex ICS-3000 workstation (Dionex, Amsterdam, The Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 × 2 mm; Dionex). The analytical separation was performed at a flow rate of 0.25 mL min⁻¹ using a complex gradient of eluents A (100 mM NaOH), B (600 mM NaOAc in 100 mM NaOH), C (Milli-Q water), and D (50 mM NaOAc). The gradient started with 10% A, 85% C, and 5% D in 25 min to 40% A, 10% C, and 50% D, followed by a 35 min gradient to 75% A and 25% B, directly followed by 5 min washing with 100% B and reconditioning for 7 min with 10% A, 85% B, and 5% D. External standards of lactose, glucose, and fructose were used to calibrate for the corresponding sugars. For determination of glucosylated lactose compounds with a degree of polymerization (DP) of 3, maltotriose was used as external standard.

Semipreparative HPAEC-PAD samples were applied in a 4 mg mL⁻¹ concentration in 250 μL injections on an ICS-5000 system, equipped with an ICS-5000 PAD detector, using a CarboPac PA-1 column (250 × 9 mm; Dionex). Fractions were manually collected and immediately neutralized with 20% acetic acid, followed by desalting over a CarboGraph SPE column (Alltech, Breda, The Netherlands).

MALDI-TOF Mass Spectrometry. Molecular masses of the compounds in the reaction mixtures were determined by MALDI-TOF mass spectrometry on an Axima Performance mass spectrometer (Shimadzu Kratos Inc., Manchester, U.K.), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Ion-gate cutoff was set to *m/z* 200 Da, and sampling resolution was software-optimized for *m/z* 1500 Da. Samples were prepared by mixing 1 μL with 1 μL of aqueous 10 mg mL⁻¹ 2,5-dihydroxybenzoic as matrix solution on the target plate.

NMR Spectroscopy. The structures of oligosaccharides of interest were elucidated by 1D and 2D ¹H–¹H NMR and 2D ¹H–¹³C NMR. A Varian Inova 500 spectrometer (NMR center, University of Groningen) was used at a probe temperatures of 25 °C. The aliquot samples were exchanged twice with 600 μL of 99.9 atom % D₂O (Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate freeze-drying and then dissolved in 0.65 mL of D₂O, containing internal acetone (δ(¹H) 2.225 ppm; δ(¹³C) 31.08 ppm). In the 1D ¹H NMR experiments, the data were recorded at 16k complex data points, and the HOD signal was suppressed using a WET1D pulse. In the 2D ¹H–¹H NMR COSY experiments, data were recorded at 4800 Hz for both directions at 4k complex data points in 256 increments. 2D ¹H–¹H NMR TOCSY data were recorded with 4000 Hz at 30, 60, 100, 150, and 200 ms spinlock times in 200 increments. In the 2D ¹H–¹H NMR ROESY, spectra were recorded with 4800 Hz at a mixing time of 300 ms in 256 increments of 4000 complex data points. MestReNova 9.1.0 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process NMR spectra, using Whittaker Smoother baseline correction.

RESULTS

Mutant Gtf180-ΔN Enzymes and Transglycosylation of Lactose.

The results of the docking experiments with lactose in a model of the glucosyl-enzyme intermediate of Gtf180-ΔN from *Lactobacillus reuteri* 180 showed that,¹⁸ within the selection of 34 productive poses, all three lactose transglycosylation types observed experimentally (F1–F3) were represented. In addition, some poses represented glycosylation types not identified experimentally. The highest number of poses favored (α1→3) elongation at the non-reducing end, followed by (α1→2) elongation at the reducing end, and then (α1→6) elongation at the reducing end (including the highest scoring pose) or (α1→4) elongation at the nonreducing end. The general picture emerging from the productive poses was that residues N1029, W1065, and Q1140 are in close proximity to, and are able to make hydrogen bond interactions (N1029, W1065, Q1140) or hydrophobic stacking interactions (W1065) with, the acceptor substrate lactose. Figure 1 shows three poses corresponding to transglycosylation scenarios yielding compounds F1–F3, which are in a position similar to that of bound maltose in previous work.¹⁸

A collection of 23 Gtf180-ΔN mutants with single amino acid residue changes at the N1029, W1065, and Q1140

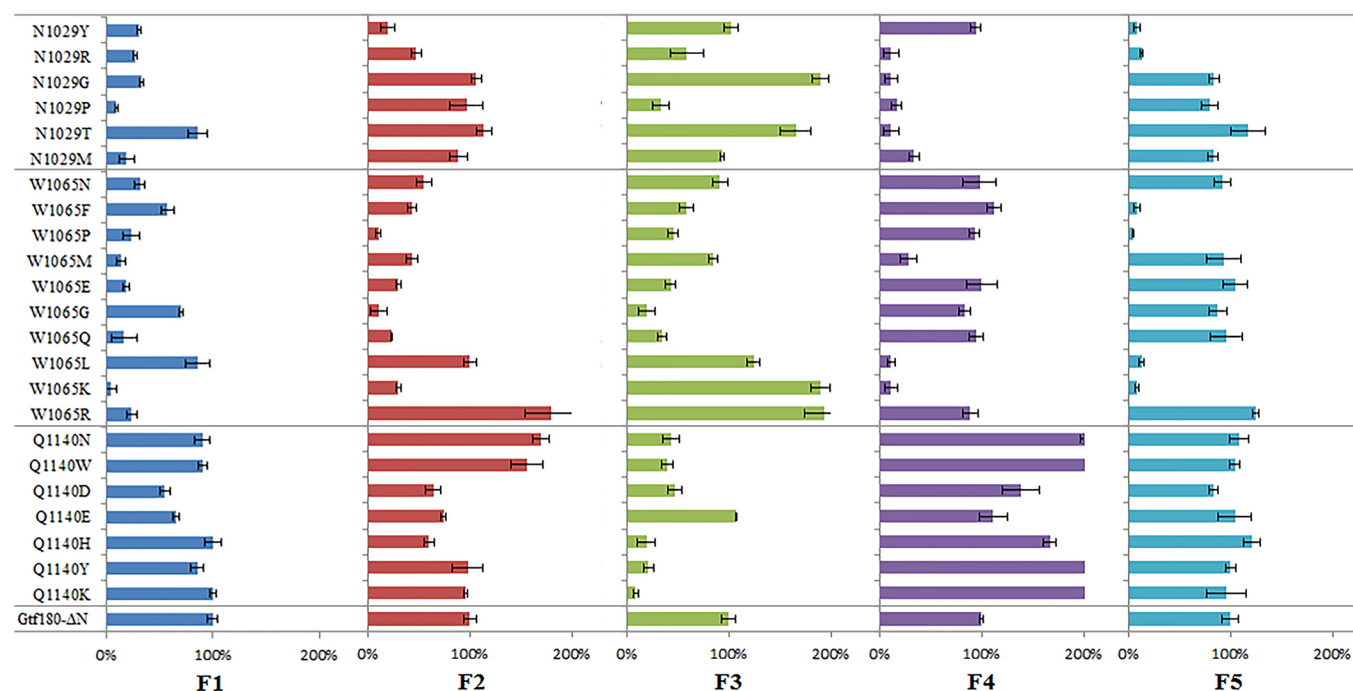


Figure 2. Effects of mutations in residues N1029, W1065, and Q1140 on the synthesis of structures F1–F5 in the GL34 mixtures, relative to wild-type Gtf180- Δ N (100%). Reactions were carried out with 0.5 M sucrose and 0.3 M lactose, catalyzed by 1 U mL⁻¹ of these enzymes at 37 °C for 24 h. The experiments were carried out in duplicate.

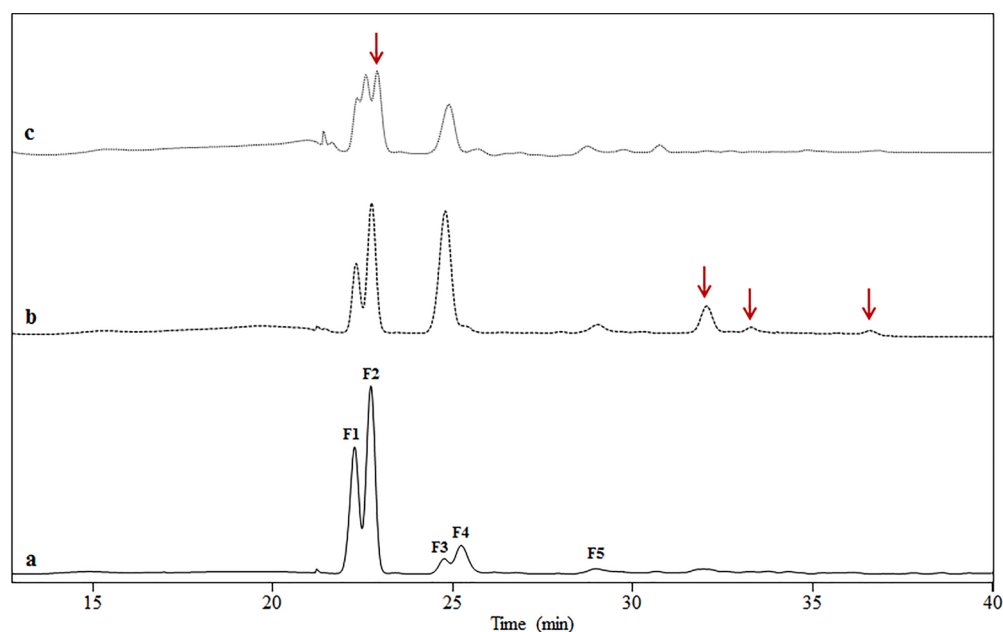


Figure 3. HPAEC-PAD profiles of lactose-derived oligosaccharide products in incubation mixtures with (a) Gtf180- Δ N wild type and corresponding mutants (b) N1029G and (c) W1065M using 1 U mL⁻¹ (total activity) at 37 °C in 25 mM sodium acetate buffer (pH 4.7) with 1 mM CaCl₂ for 24 h. The red arrows indicate new peaks in the reaction mixture catalyzed by mutants of Gtf180- Δ N, identified as G1–G4.

positions had been constructed previously^{30,31} and was used for analysis of transglycosylation reactions with lactose as acceptor substrate. All of the reactions were carried out with 0.5 M sucrose and 0.3 M lactose, catalyzed by 1 U mL⁻¹ of the corresponding purified Gtf180- Δ N mutant or wild-type enzymes for 24 h. The incubation mixtures were analyzed by HPAEC-PAD profiling for a semiquantitative evaluation of the formation of the F1–F5 compounds. Mutations in these residues resulted in clear changes in the F1–F5 amounts

synthesized. Figure 2 shows the percentages of the individual F1–F5 structures synthesized by the mutant enzymes in comparison to the 100% values for the Gtf180- Δ N wild type. Each enzyme was used at 1 U mL⁻¹, but the total amount of the GL34 mixture synthesized was mostly less than that by Gtf180- Δ N, except for mutants Q1140W, Q1140N, and N1029T (Figure 2).

Mutants of Residue Q1140. HPAEC-PAD analysis of the Q1140 mutants showed a clear decrease of F3 with an (α 1 \rightarrow 3)

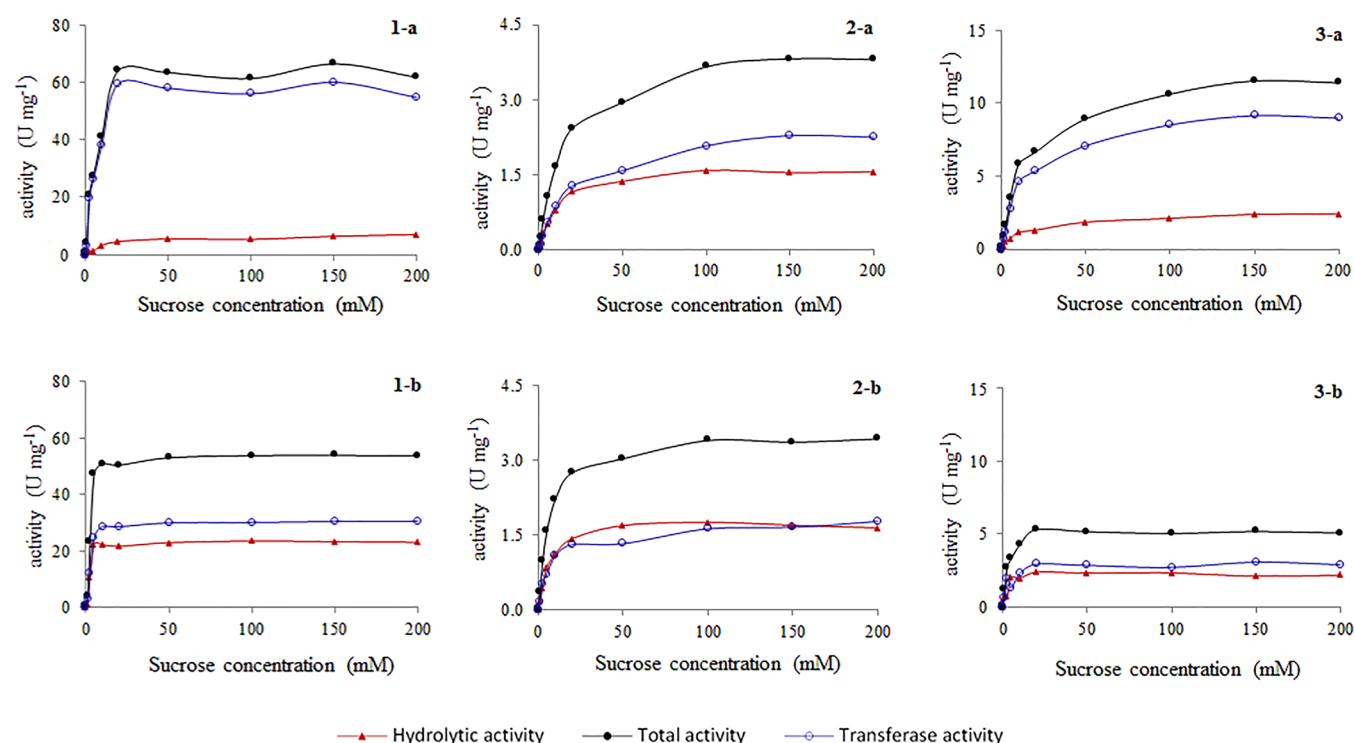


Figure 4. Influence of sucrose concentration on initial activities (hydrolytic, transferase, and total activity) of different enzymes: (1) Gtf180-ΔN; (2) W1065M, and (3) N1029G when using (a) sucrose as donor substrate and 200 mM lactose as acceptor substrate or (b) only sucrose as substrate, with 5 μg of the protein of the corresponding enzymes at 37 °C in 25 mM sodium acetate/1 mM CaCl₂ buffer, pH 4.7. Experiments were carried out in duplicate.

Table 1. Kinetic Analysis of the Activities of Gtf180-ΔN and Mutants Derived Using Only Sucrose or both Sucrose and Lactose as Substrates^a

enzyme	substrate	hydrolytic activity			transferase activity			total activity		
		K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
Gtf180-ΔN	lactose and sucrose	13.3 ± 1.1	32.9 ± 0.2	2.5	5.6 ± 0.2	294.8 ± 3.2	52.6	6.0 ± 0.4	326.2 ± 4.2	54.4
	sucrose	2.5 ± 0.3	118.1 ± 1.3	47.2	2.9 ± 0.2	153.3 ± 3.8	52.9	2.7 ± 0.2	271.4 ± 3.1	100.5
N1029G	lactose and sucrose	13.5 ± 0.3	15.2 ± 0.5	1.1	13.6 ± 0.1	58.2 ± 2.4	4.3	13.7 ± 1.3	73.3 ± 2.5	5.4
	sucrose	2.6 ± 0.2	14.2 ± 0.6	5.6	3.0 ± 0.1	18.2 ± 1.8	6.1	2.7 ± 0.0	32.7 ± 1.2	12.1
W1065M	lactose and sucrose	10.4 ± 0.9	3.7 ± 0.6	0.4	19.3 ± 0.4	5.4 ± 0.3	0.3	15.1 ± 1.1	8.8 ± 0.4	0.6
	sucrose	6.0 ± 0.7	3.9 ± 0.3	0.6	6.9 ± 0.8	3.7 ± 0.9	0.5	6.2 ± 0.6	7.5 ± 0.5	1.2

^a K_m values calculated for sucrose. Experiments were carried out in duplicate.

linkage and an increase in F4 with (α1→4)/(α1→2) linkages in comparison with the profiles of the wild type enzyme (Figure 2). Compound F5 with both (α1→2) and (α1→3) linkages remained relatively constant for all studied Q1140 mutants. Similarly, F1 with an (α1→4) linked Glc was hardly affected by these mutations, except for mutants Q1140E and Q1140D, which showed a decrease in F1. Structure F2 with an (α1→2)-linked Glc was increased in mutants Q1140W and Q1140N but was decreased in mutants Q1140H, Q1140E, and Q1140D.

Mutants of Residue W1065. Amino acid changes at W1065 exhibited more diverse effects on the glucosylated-lactose product profile (Figure 2). Most of the W1065 mutants displayed a decrease in all GL34 compounds. F2 with an (α1→2) linked Glc and F4 with (α1→2)- and (α1→4)-linked Glc were most strongly affected (Figure 2). A decrease in F2 was found for most of the W1065 mutants, except for W1065L and

W1065R. Three substitutions of Trp1065 with Arg, Lys, and Asn resulted in a clear increase in the amount of F3, with an (α1→3)-linked Glc. In the reaction with maltose as acceptor substrate, the same mutants also synthesized increased levels of oligosaccharides with an (α1→3)-linked Glc.³⁵ The positive charge of Arg and Lys may be an important determinant for favoring (α1→3) linkage formation. The amounts of F4 and F5 DP4 compounds were very minor in the profiles of mutants W1065L and W1065K (Figure 2).

Mutants of Residue N1029. Mutants of N1029 showed a clear decrease in F4 with (α1→4)- and (α1→2)-linked Glc moieties; the most significant reduction was caused by replacing Asn1029 with Arg, Gly, Pro, and Thr residues (Figure 2). Except for the residue Arg, the three other amino acid residues are much smaller in size than Asn, which may result in significant changes in acceptor substrate binding by this glucansucrase. The relative levels of F1, with an (α1→4)

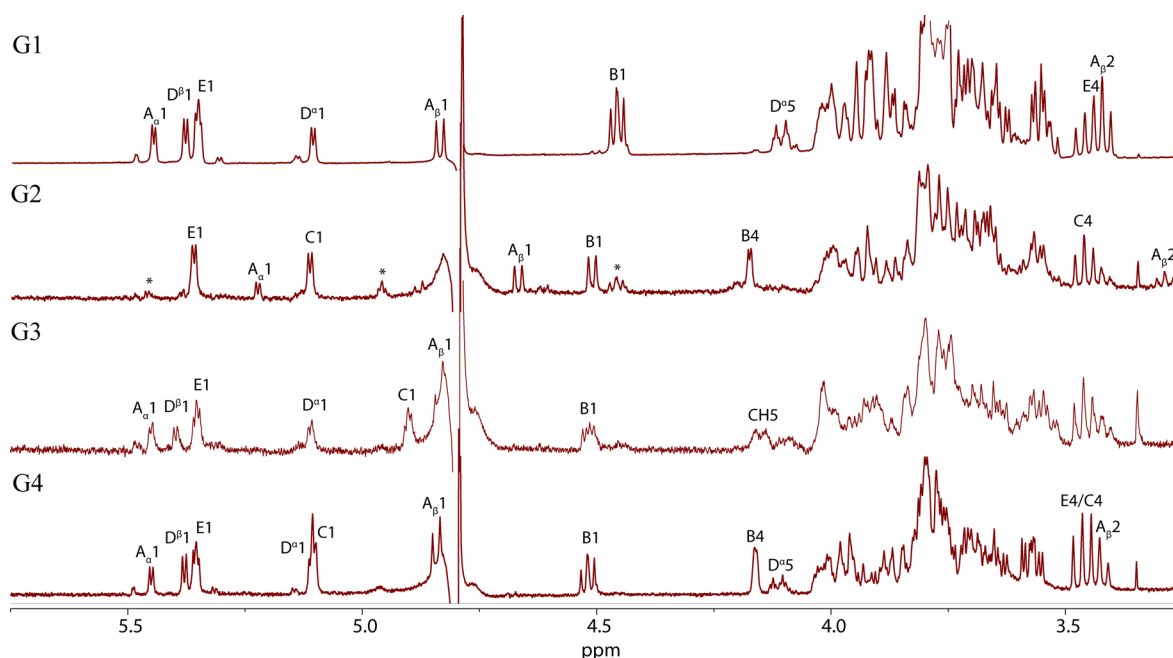


Figure 5. 500 MHz 1D ^1H NMR spectra of G1–G4 fractions from the reaction mixture with Gtf180- ΔN N1029G (see Figure S1), recorded at 25 $^\circ\text{C}$ in D_2O . Anomeric signals of each fraction were labeled according to the legends of corresponding structures indicated in Scheme 1.

linked Glc, were decreased in most of the N1029 mutants (Figure 2). Substitutions of Asn with Gly or Thr resulted in clear increases in the amount of F3, with an $(\alpha 1 \rightarrow 3)$ -linked Glc. N1029 mutagenesis thus has a clear effect on linkage specificity of lactose-derived oligosaccharides, with fewer $(\alpha 1 \rightarrow 4)$ linkages and more $(\alpha 1 \rightarrow 3)$ linkages.

Among all studied mutants, only the product profiles of N1029P, N1029G, and W1065M with sucrose/lactose showed new peaks in comparison with that of Gtf180- ΔN wild type (Figure 3). The new peaks in the N1029P (not shown) and N1029G profiles appear at the same position in the HPAEC-PAD profile, but they were more intense in the N1029G profile. Mutants N1029G and W1065M were selected for further biochemical analysis. The new transglycosylated lactose products of N1029G were structurally characterized. The W1065M products were not characterized further.

Biochemical Analysis of the Glucansucrase Reaction with Sucrose and Lactose. The N1029G and W1065M mutants were studied in comparison with Gtf180- ΔN wild type, in the reactions with lactose as acceptor substrate or with only sucrose. All tested (mutant) glucansucrases displayed Michaelis–Menten type kinetics for both the hydrolysis and transferase activities at sucrose concentrations between 0.1 and 200 mM (Figure 4). Mutations at the N1029 and W1065 positions clearly resulted in reduced activities. The K_m , k_{cat} , and catalytic efficiency values were calculated accordingly (Table 1). The K_m values for sucrose in the Gtf180- ΔN wild type and the mutants N1029G and W1065M reactions with only sucrose were significantly lower than in the reactions with lactose plus sucrose. The presence of lactose molecules around the substrate binding site apparently results in a more limited access for sucrose and a reduced binding affinity of Gtf180- ΔN and its mutants for sucrose. The presence of sucrose plus lactose as acceptor substrate resulted in a strong reduction of hydrolytic activity for Gtf180- ΔN and an increase in transferase activity for Gtf180- ΔN and mutant N1029G, in comparison to activity with sucrose alone. The k_{cat} value and

hydrolytic efficiency k_{cat}/K_m of Gtf180- ΔN were suppressed strongly with lactose as acceptor substrate. However, the transferase efficacies k_{cat}/K_m of this enzyme with and without lactose were similar at about $52 \text{ s}^{-1} \text{ mM}^{-1}$. Analysis of the N1029G mutant showed that replacing Asn by Gly caused a clear decrease in activity (hydrolytic and total activity) in comparison with the wild type enzyme. Similarly to Gtf180- ΔN wild type, the sucrose hydrolytic efficiency of this mutant is lower in the presence of lactose. Although the k_{cat} value of N1029G with lactose plus sucrose is significantly higher than that with only sucrose, 58.2 vs 18.2 s^{-1} , the transferase efficacy k_{cat}/K_m values are not significantly different. Kinetic analysis of mutant W1065M showed that both its K_m and k_{cat} values were affected significantly, resulting in a very low catalytic efficiency (Table 1).

Structural Characterization of Transglycosylated Products Synthesized by Gtf180- ΔN N1029G. Mutant N1029G was used in a larger scale incubation with 0.5 M sucrose and 0.3 M lactose, followed by product fractionation by HPAEC-PAD on a semipreparative column. Collected fractions were analyzed by MALDI-TOF mass spectrometry (data not shown). Fractions G1 and G2 each consisted of a major component with four hexose units (DP4; m/z 689). Fractions G3 and G4 each had a major component with five hexose units (DP5; m/z 851) (Figure S1). The structures of these compounds were elucidated by 1D ^1H NMR, 2D ^1H – ^1H NMR, and ^1H – ^{13}C NMR.

Fraction G1. Compound G1 is composed of four hexose residues: namely, A and B (glucosyl and galactosyl residues from original lactose, respectively) and D and E (transferred glucosyl residues from sucrose). The ^1H anomeric signals of fraction G1 were revealed by 500 MHz 1D ^1H NMR spectrum as following δ 5.447 (A_α H-1), δ 4.838 (A_β H-1), δ 4.469/4.452 (B H-1), δ 5.108 (D^α H-1), δ 5.381 (D^β H-1), and δ 5.358/5.349 (E H-1) (Figure 5). The splitting of the anomeric signals B H-1, D H-1, and E H-1 is influenced by the α/β configuration of the reducing residue A. All nonanomeric

Table 2. ^1H and ^{13}C Chemical Shifts of the Glucosylated Lactose Derivatives, Measured at 25 °C in D_2O^a

	Lac		G1		G2 ^1H	G3 ^1H	G4	
	^1H	^{13}C	^1H	^{13}C			^1H	^{13}C
A $_{\alpha}$ 1	5.222	92.8	5.447	90.0	5.225	5.450	5.450	89.9
A $_{\alpha}$ 2	3.58	72.4	<u>3.70</u>	<u>79.3</u>	3.59	<u>3.69</u>	<u>3.71</u>	<u>79.6</u>
A $_{\alpha}$ 3	3.83	72.5	3.97	70.9	3.85	3.95	3.97	72.8
A $_{\alpha}$ 4	3.66	79.8	<u>3.71</u>	<u>79.3</u>	<u>3.68</u>	3.70	<u>3.74</u>	<u>80.5</u>
A $_{\alpha}$ 5	3.95	71.2	4.01	72.5	3.97	4.05	4.06	70.6
A $_{\alpha}$ 6a	3.87	61.5	3.89	60.8			3.88	61.2
A $_{\alpha}$ 6b	3.84							
A $_{\beta}$ 1	4.662	96.9	4.838	96.7	4.670	4.828	4.843	97.0
A $_{\beta}$ 2	3.287	<u>75.0</u>	<u>3.43</u>	<u>78.9</u>	3.30	<u>3.42</u>	<u>3.43</u>	<u>79.0</u>
A $_{\beta}$ 3	3.63	75.4	3.73	76.3	3.63	3.74	3.76	73.8
A $_{\beta}$ 4	3.66	79.8	<u>3.68</u>	<u>79.3</u>	<u>3.66</u>	<u>3.68</u>	<u>3.69</u>	<u>79.5</u>
A $_{\beta}$ 5	3.60	75.8	3.60	75.3	3.61	3.60	3.61	75.5
A $_{\beta}$ 6a	3.95	61.6	3.96	60.9	3.96	3.83	3.94	61.2
A $_{\beta}$ 6b	3.80		3.80	60.9	3.81	3.97	3.80	
B1	4.447	104.4	4.469/4.452	103.6	4.511	4.525/4.508	4.527/4.512	104.1
B2	3.54	72.3	3.55	72.0	3.68	3.59	3.70	70.8
B3	3.66	73.7	3.67	72.3	<u>3.78</u>	3.76	<u>3.77</u>	<u>78.6</u>
B4	3.92	69.8	3.92	69.5	<u>4.18</u>	<u>4.02</u>	<u>4.16</u>	66.0
B5	3.72	76.4	3.94		3.72	3.68	3.69	75.9
B6a	3.80	62.2	3.76	61.6	3.80	3.80	3.80	62.0
B6b	3.75		3.71	61.6		3.70	3.74	
C1					5.114	4.907/4.896	5.111/5.103	96.7
C2					3.68	3.54	3.58	72.3
C3					<u>3.93</u>	3.75	3.78	74.1
C4					3.66	3.47	3.46	70.2
C5					4.00	<u>4.16</u>	4.02	72.7
C6a					3.81	3.80	3.84	61.2
C6b					3.78		3.76	
D $^{\alpha}$ 1			5.108	97.2		5.110	5.102	96.7
D $^{\alpha}$ 2			3.64	75.1		3.65	3.59	74.5
D $^{\alpha}$ 3			<u>3.93</u>	<u>80.6</u>		<u>3.94</u>	<u>3.93</u>	<u>80.6</u>
D $^{\alpha}$ 4			3.49	70.4		3.48	3.46	70.3
D $^{\alpha}$ 5			<u>4.02</u>	72.4		4.00	3.96	72.6
D $^{\alpha}$ 6a			3.87	60.9		3.80	3.80	61.2
D $^{\alpha}$ 6b			3.80	60.9			3.76	
D $^{\beta}$ 1			5.381	98.8		5.399	5.379	98.6
D $^{\beta}$ 2			3.53	72.1		3.64	3.64	71.0
D $^{\beta}$ 3			<u>3.79</u>	<u>80.7</u>		<u>3.90</u>	<u>3.89</u>	<u>80.9</u>
D $^{\beta}$ 4			3.47	70.3		3.46	3.47	70.2
D $^{\beta}$ 5			<u>4.11</u>	72.1		<u>4.10</u>	<u>4.11</u>	72.1
D $^{\beta}$ 6a			3.91	60.9		3.81	3.80	61.2
D $^{\beta}$ 6b			3.80	60.9				
E1			5.358/5.349	100.0	5.361	5.356/5.347	5.358/5.350	100.2
E2			3.54	72.1	3.57	3.57	3.57	72.4
E3			3.75	73.9	3.75	3.75	3.76	73.9
E4			3.45	70.2	3.48	3.45	3.44	70.4
E5			<u>4.02</u>	72.6	<u>4.02</u>	<u>4.02</u>	<u>4.02</u>	72.7
E6a			3.85	61.0	3.87	3.83	3.82	61.2
E6b			3.80	61.5	3.80			

^aChemical shifts that are key in the structural determination are underlined.

proton resonances were assigned by using 2D ^1H – ^1H TOCSY and 2D ^1H – ^{13}C HSQC (Table 2 and Figure S2b). At the glucosyl residue A, strong downfield shifts were detected for A $_{\alpha}$ H-2 at δ 3.70 ($\Delta\delta$ +0.12 ppm), A $_{\alpha}$ C-2 at δ 79.3 ($\Delta\delta$ +6.90 ppm), A $_{\beta}$ H-2 at δ 3.43 ($\Delta\delta$ +0.14 ppm) and A $_{\beta}$ C-2 δ 78.9 ($\Delta\delta$ +3.90 ppm), suggesting the occurrence of substituted O-2 of this residue.³⁶ The 2D ROESY double inter-residual cross-peaks D $^{\alpha}$ H-1/A $_{\alpha}$ H-2 and D $^{\beta}$ H-1/A $_{\beta}$ H-2 confirmed this 2-

substitution of residue A (Figure S2c). The high anomeric resonance value of A $_{\alpha}$ H-1 at δ 5.447 ppm stems from this 2-substituted reducing α -D-Glcp unit.¹¹ Additionally, significant downfield shifts of D $^{\alpha}$ H-3 at δ 3.93 ($\Delta\delta$ +0.10 ppm), D $^{\alpha}$ C-3 at δ 80.6 ($\Delta\delta$ +8.10 ppm), D $^{\beta}$ H-3 at δ 3.89 ($\Delta\delta$ +0.26 ppm), and D $^{\beta}$ C-3 at δ 80.7 ($\Delta\delta$ + 5.30 ppm) are indicative of a substitution at O-3 of this residue. This substitution is supported by the 2D ROESY inter-residual cross-peak between

E H-1 and D^β H-3 (Figure S2c). The inter-residual interaction between B H-1 and A_α H-4 was also detected in the ROESY spectrum. When all data are combined, the structure of tetrasaccharide compound G1 was determined to be α-D-Glcp-(1→3)-α-D-Glcp-(1→2)-[β-D-Galp-(1→4)]-D-Glcp (Scheme 1).

Fraction G2. Compound G2 is composed of 4 hexose residues, including A and B (glucosyl and galactosyl residues from original lactose, respectively) and C and E (transferred glucosyl residues from sucrose). The ¹H NMR spectrum showed five anomeric signals at δ 5.225 (A_α H-1), δ 4.670 (A_β H-1), δ 4.511 (B H-1), δ 5.114 (C H-1), and δ 5.361 (E H-1) (Figure 5). Assignments of nonanomeric resonances of these residues were obtained by 2D ¹H–¹H TOCSY measurements (Table 2 and Figure S3b). The chemical shifts of residues A–C of this fraction were found to be highly similar to those values of fraction F3 from the GL34 mixture.²⁷ The ROESY inter-residual cross peaks C H-1/B H-3 verified O-3 substitution occurring at residue B (Figure S3c). Moreover, at residue C, the chemical shift of H-3 was shifted strongly to δ 3.93 (Δδ + 0.13 ppm) in reference to that of the same residue from compound F3,²⁷ indicating the substitution at O-3 of this residue. This speculation was confirmed by the 2D ROESY inter-residual cross-peak between E H-1 and C H-3 (Figure S3c). The 3-substitution at residues B and C has a strong influence on the chemical shift value of residue B H-4 at δ 4.18 (Δδ + 0.26 ppm). The ROESY spectrum also showed inter-residual correlations between C H-1 and B H-3 and between B H-1 and A H-4 (Figure S3c). These data resulted in the identification of compound G2 as a tetrasaccharide with the structure α-D-Glcp-(1→3)-α-D-Glcp-(1→3)-β-D-Galp-(1→4)-α-D-Glcp (Scheme 1).

Fraction G3. Compound G3 consists of five hexose residues: A and B (glucosyl and galactosyl residues from original lactose, respectively) and C–E (transferred glucosyl residues from sucrose). Seven anomeric signals of this compound were detected by 1D ¹H NMR to be at δ 5.450 (A_α H-1), δ 4.828 (A_β H-1), δ 4.525/4.508 (B H-1), δ 4.907/4.896 (C H-1), δ 5.110 (D^α H-1), δ 5.399 (D^β H-1), and δ 5.356/5.347 (E H-1) (Figure 5). Three other anomeric signals from this fraction G3 were also detected, marked with asterisks in the 1D ¹H NMR profile; they were too minor to be identified (Figure S4a). The splitting of the anomeric signals B H-1, D H-1, and E H-1 was due to the influence of the α/β configuration of reduction residue A. All nonanomeric proton resonances were assigned by using 2D ¹H–¹H TOCSY (Table 2 and Figure S4b). The set of ¹H and ¹³C chemical shifts of residues A–C correspond to the values of these residues occurring in compound F4 of the GL34 mixture.²⁷ The 2D ROESY doubled inter-residual cross-peaks C H-1/B H-4 together with a strong downfield of residue B H-4 at δ 4.02 (Δδ + 0.10 ppm) indicates the occurrence of an O-4 substitution at the residue B (compare structure F1 in Pham et al. 2017).²⁷ Strong downfield shifts of the residue A with A_α H-2 at δ 3.69 (Δδ + 0.11 ppm) and A_β H-2 at δ 3.42 (Δδ + 0.13 ppm) were found as indications of substituted O-2 at this residue. This 2-substitution of residue A was verified by the 2D ROESY doubled inter-residual cross-peaks D^β H-1/A_β H-2 (Figure S4c). The resonances of residue D^α H-3 and D^β H-3 were shifted significantly to δ 3.94 (Δδ + 0.14 ppm) and δ 3.90 (Δδ + 0.15 ppm), respectively, in comparison to residue D of compound F4,²⁷ reflecting a –(1→3)-α-D-Glcp– unit. The 2D ROESY inter-residual cross-peak between E H-1 and D^β/D^α

H-3 supported this O-3 substitution (Figure S4c). These data lead to the structure of this compound G3 as α-D-Glcp-(1→3)-α-D-Glcp-(1→2)-[Glcp-(1→4)-β-D-Galp-(1→4)]-D-Glcp (Scheme 1).

Fraction G4. Compound G4 consists of five hexose residues, namely A and B (glucosyl and galactosyl residues from original lactose, respectively) and C–E (transferred glucosyl residues from sucrose). The 1D ¹H NMR spectrum of fraction G4 showed seven anomeric signals at δ 5.450 (A_α H-1), δ 4.843 (A_β H-1), δ 4.527/4.512 (B H-1), δ 5.111/5.103 (C H-1), δ 5.102 (D^α H-1), δ 5.379 (D^β H-1), and δ 5.358/5.350 (E H-1) (Figure 5). The splitting of the anomeric signals B H-1, C H-1, D H-1, and E H-1 is caused by α/β configuration of the reduction residue A. The other nonanomeric proton resonances were determined by 2D ¹H–¹H TOCSY, and the carbon chemical shifts were correlated to ¹³C in the 2D ¹H–¹³C HSQC spectrum (Table 2 and Figure S5c). The set of ¹H and ¹³C chemical shifts of residues A–C matched very well with the values of these residues in compound F5 of the GL34 mixture, although with slight shifts,²⁷ suggesting the occurrence of a 3-substituted galactosyl residue and 2-substituted glucosyl residue. The strong downfield shifts of residue B H-3 at 3.77 (Δδ + 0.11 ppm) and C-3 at 78.6 (Δδ + 4.9 ppm) and the ROESY inter-residual cross peaks C H-1/B H-3 verify the α-D-Glcp-(1→3)– linkage (Figure S5c). Strong downfield shifts of residue A with A_α H-2 at δ 3.71 (Δδ + 0.13 ppm), A_α C-2 at δ 79.6 (Δδ + 6.93 ppm), A_β H-2 at δ 3.43 (Δδ + 0.14 ppm), and A_β C-2 δ 79.0 (Δδ + 3.91 ppm) were detected as indications for a substituted O-2 of residue A. This 2-substitution of residue A is confirmed by the 2D ROESY doubled inter-residual cross-peaks D^β H-1/A_β H-2 and D^α H-1/A_α H-2 (Figure S5c). The resonances of residues D^α H-3 and D^β H-3 shifted significantly to values of δ 3.93 (Δδ + 0.13 ppm) and δ 3.89 (Δδ + 0.14 ppm), respectively, in comparison to that residue of compound F5,²⁷ indicating the occurrence of O-3 substitution at residue D. This substitution is supported by the 2D ROESY inter-residual cross-peak between E H-1 and D^β/D^α H-3. These data determined the structure of G4 to be α-D-Glcp-(1→3)-α-D-Glcp-(1→2)-[α-D-Glcp-(1→3)-β-D-Galp-(1→4)]-D-Glcp (Scheme 1).

DISCUSSION

Residues surrounding the glucanucrase active site have been subjected to several mutagenesis studies aiming to identify the structural determinants of product size and linkage specificity in these enzymes.^{21,23,37,30,31,38,39} The three residues (N1029, W1065, and Q1140) targeted in this work have been studied before, but in reactions with different acceptor substrates. Notably, all three residues are fully conserved within glucanucrases, and they play an important role in the transglycosylation reaction. Residue N1029 in domain A of Gtf180-ΔN was previously shown to be critical for linkage specificity and activity. Meng et al. reported the essential role of N1029 in acceptor substrate binding by Gtf180-ΔN.³⁰ Regarding α-glucan synthesis from sucrose, N1029 mutants tended to decrease the transglycosylation/hydrolysis ratio and to increase the relative amount of (α1→3) linkages in the products. This was attributed to the fact that N1029 is involved in the hydrogen bond network to bound acceptor substrates.^{18,30} In addition, residue W1065 appeared to be critical for the activity of this enzyme.³¹ The stacking interactions of this aromatic residue with acceptor substrates

bound at subsites +1 and +2 were shown to be required for polysaccharide synthesis.³¹ Mutation of this W1065 residue also affected linkage specificity in polysaccharide and oligosaccharide synthesis in reactions using sucrose or sucrose (donor) plus maltose (acceptor) as substrates.^{18,21,31} Finally, the importance of residue Q1140 was shown in a study where Gtf180- Δ N was used to transglycosylate steviol glycosides.¹⁵

Also with lactose as an acceptor substrate, mutation of these amino acid residues (N1029, W1065, and Q1140) showed clear effects on the activity as well as the linkage specificity of Gtf180- Δ N. First, replacement of N1029 with Gly or Thr facilitated these Gtf180- Δ N mutants to synthesize (α 1 \rightarrow 3) glucosylated lactose derivatives (Figure 2). This resulted in a strong reduction in the synthesis of (α 1 \rightarrow 4) glucosidic linkages containing compounds (F1 and F4) by mutant N1029G in comparison to the wild-type enzyme (Figure 2). Similar effects were observed in studies where maltose was used as acceptor substrate,³⁵ or even when non-carbohydrate compounds were used as acceptor substrates.⁶ The crystal structure of GTF180- Δ N in complex with maltose revealed that N1029 is involved in a hydrogen bond network with the nonreducing end glucosyl moiety of maltose in subsite +1, making direct and indirect hydrogen bonds with its C3 and C4 hydroxyl groups.¹⁸ Mutant N1029G, when it acts on lactose as an acceptor substrate, added an (α 1 \rightarrow 3)-linked Glc moiety to compounds F2–F5 of the GL34 mixture to synthesize G1–G4 (Scheme 1). However, under the incubation conditions tested, the amounts of the novel compounds G1–G4 were relatively low in comparison with F1–F5; optimization of these reactions clearly is required in order to increase the yield of this (α 1 \rightarrow 3) linkage containing a lactose-derivative mixture.

Second, with most of the W1065 mutants we observed a decrease in the amounts of F1–F5 synthesized (Figure 2). This may be explained by a loss of the aromatic stacking interaction with the acceptor substrate lactose, similar to what has been observed in studies using sucrose or sucrose plus maltose.³¹ It is interesting to note that a wild-type glucansucrase is able to catalyze linear (α 1 \rightarrow 2)-glycosylation, albeit with a “non-natural” acceptor substrate. The related and highly homologous branching sucrases also synthesize (α 1 \rightarrow 2) linkages, but only with dextran as an acceptor substrate, and with a nonaromatic residue replacing the tryptophan at position 1065.^{29,40,28} Moreover, they only form (α 1 \rightarrow 2) branch points instead of linear (α 1 \rightarrow 2) linkages. W1065 mutants still synthesized products with (α 1 \rightarrow 2) linkages (F2, F4), although the amounts were decreased. Mutant W1065M was almost completely inactive, which is in agreement with an earlier study.³¹ Together, our results show that W1065 is essential for the transglycosylation of lactose.

Third, Q1140 mutant enzymes showed minor changes in their GL34 mixture profiles. Only compound F3 with an (α 1 \rightarrow 3)-linked Glc moiety was clearly reduced. Residue Q1140, together with residues N1411 and D1458, lines the pocket-shaped cavity of Gtf180 in which the glucosyl moiety of sucrose binds (subsite –1) but is also near subsite +2.¹⁸ Mutations of Q1140 change the surface shape and/or local charge and thereby may affect the affinity for and/or orientation of bound acceptor molecules, explaining the observed changes in linkage specificity.

The results of the docking experiment of lactose in Gtf180- Δ N do not fully explain the transglycosylation types and the experimentally observed preferences but do provide insights into how lactose may bind at acceptor binding subsites +1 and

+2. Reflecting the variety in linkage types observed experimentally for lactose transglycosylation by wild-type Gtf180- Δ N, the set of obtained poses showed a large variation of orientations, which may be related to the fact that the acceptor binding region of Gtf180- Δ N is quite wide and open. The observation that product profiles of N1029, W1065, and Q1140 mutants are affected (with respect to wild type) agrees with the fact that, in the docking results, lactose interacts with these residues when bound for transglycosylation. Further insights into the linkage specificity determinants of Gtf180- Δ N acting on lactose as acceptor substrate may be provided by a crystal structure of Gtf180- Δ N in complex with lactose, but attempts to obtain such a complex have not been successful so far.

Recently we reported that the GL34 mixture synthesized by Gtf180- Δ N shows the potential to shift microbiota composition: it specifically stimulated growth of bifidobacteria, particularly *B. adolescentis*.⁴¹ This novel GL34 oligosaccharide mixture, synthesized from cheap and abundantly available lactose and sucrose, thus (potentially) has synbiotic properties toward *B. adolescentis*. Glucansucrases are highly interesting glucosylating enzymes that are relatively easy to produce, highly active with sucrose as donor substrate, and have promising conversion degrees. Optimization of their transglycosylation reactions with galactose-containing compounds as acceptor substrates is needed to obtain higher yields of transfer products for further functional studies.

The current study identified three residues (N1029, W1065, and Q1140) that likely play a role in determining linkage specificity regarding lactose transglycosylation. The fact that all three residues are fully conserved in glucansucrases underpins their importance and expands the possibilities for understanding the synthesis of lactose-derived oligosaccharides by glucansucrases (and their mutants). Ultimately, further tailoring and optimization of their synthesis may lead to desired products for further applications.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04486.

1D ¹H NMR spectrum, 2D ¹H–¹H TOCSY spectrum, and 2D ¹H–¹H ROESY spectrum of compounds G1–G4 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail for L.D.: L.Dijkhuizen@rug.nl.

ORCID

Hien Pham: 0000-0001-8482-3482

Present Addresses

[§]L.D.: CarbExplore Research BV, Zernikepark 12, 9747 AN Groningen, The Netherlands.

^{||}S.S.v.L.: Laboratory Medicine, University Medical Center Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The work was financially supported by the University of Groningen/Campus Fryslân, FrieslandCampina, and The University of Groningen.

ABBREVIATIONS USED

Gtfs, glucansucrases; HPAEC-PAD, high-pH anion exchange chromatography-pulse amperometric detection; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NMR, nuclear magnetic resonance

REFERENCES

- (1) Monchois, V.; Willemot, R. M.; Monsan, P. Glucansucrases: Mechanism of action and structure-function relationships. *FEMS Microbiol. Rev.* **1999**, *23* (2), 131–151.
- (2) Lombard, V.; Golaconda Ramulu, H.; Drula, E.; Coutinho, P. M.; Henrissat, B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* **2014**, *42*, D490–D495.
- (3) Leemhuis, H.; Pijning, T.; Dobruchowska, J. M.; van Leeuwen, S. S.; Kralj, S.; Dijkstra, W. B.; Dijkhuizen, L. Glucansucrases: three-dimensional structures, reactions, mechanism, α -glucan analysis and their implications in biotechnology and food applications. *J. Biotechnol.* **2013**, *163* (2), 250–272.
- (4) Moulis, C.; Joucla, G.; Harrison, D.; Fabre, E.; Potocki-Veronese, G.; Monsan, P.; Remaud-Simeon, M. Understanding the polymerization mechanism of glycoside-hydrolase family 70 glucansucrases. *J. Biol. Chem.* **2006**, *281* (42), 31254–31267.
- (5) te Poele, E. M.; Valk, V.; Devlamynck, T.; van Leeuwen, S. S.; Dijkhuizen, L. Catechol glucosides act as donor/acceptor substrates of glucansucrase enzymes of *Lactobacillus reuteri*. *Appl. Microbiol. Biotechnol.* **2017**, *101* (11), 4495–4505.
- (6) Devlamynck, T.; te Poele, E. M.; Meng, X.; van Leeuwen, S. S.; Dijkhuizen, L. Glucansucrase Gtf180- Δ N of *Lactobacillus reuteri* 180: enzyme and reaction engineering for improved glycosylation of non-carbohydrate molecules. *Appl. Microbiol. Biotechnol.* **2016**, *100* (17), 7529–7539.
- (7) Yoon, S. H.; Robyt, J. F. Synthesis of acarbose analogues by transglycosylation reactions of *Leuconostoc mesenteroides* B-512FMC and B-742CB dextranases. *Carbohydr. Res.* **2002**, *337* (24), 2427–2435.
- (8) Kim, Y. M.; Yeon, M. J.; Choi, N. S.; Chang, Y. H.; Jung, M. Y.; Song, J. J.; Kim, J. S. Purification and characterization of a novel glucansucrase from *Leuconostoc lactis* EG001. *Microbiol. Res.* **2010**, *165* (5), 384–391.
- (9) Bertrand, A.; Morel, S.; Lefoulon, F.; Rolland, Y.; Monsan, P.; Remaud-Simeon, M. *Leuconostoc mesenteroides* glucansucrase synthesis of flavonoid glucosides by acceptor reactions in aqueous-organic solvents. *Carbohydr. Res.* **2006**, *341* (7), 855–863.
- (10) Monsan, P.; Remaud-Siméon, M.; André, I. Transglucosidases as efficient tools for oligosaccharide and glucoconjugate synthesis. *Curr. Opin. Microbiol.* **2010**, *13* (3), 293–300.
- (11) Côté, G. L.; Robyt, J. F. Acceptor reactions of alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355. *Carbohydr. Res.* **1982**, *111* (1), 127–142.
- (12) Gerwig, G. J.; te Poele, E. M.; Dijkhuizen, L.; Kamerling, J. P. Stevia glycosides: chemical and enzymatic modifications of their carbohydrate moieties to improve the sweet-tasting quality. *Adv. Carbohydr. Chem. Biochem.* **2016**, *73*, 1–72.
- (13) Bai, Y.; Böger, M.; Van Der Kaaij, R. M.; Woortman, J. J. A.; Pijning, T.; van Leeuwen, S. S.; van Bueren, L. A.; Dijkhuizen, L. *Lactobacillus reuteri* strains convert starch and maltodextrins into homopolysaccharides using an extracellular and cell-associated 4,6- α -Glucanotransferase. *J. Agric. Food Chem.* **2016**, *64* (14), 2941–2952.
- (14) Díez-Municio, M.; Montilla, A.; Jimeno, M. L.; Corzo, N.; Olano, A.; Moreno, F. J. Synthesis and characterization of a potential prebiotic trisaccharide from cheese whey permeate and sucrose by *Leuconostoc mesenteroides* dextranase. *J. Agric. Food Chem.* **2012**, *60*, 1945–1953.
- (15) Te Poele, E. M.; Devlamynck, T.; Jäger, M.; Gerwig, G. J.; van de Walle, D.; Dewettinck, K.; Hirsch, K. H. A.; Kamerling, J. P.; Soetaert, W.; Dijkhuizen, L. Glucansucrase (mutant) enzymes from *Lactobacillus reuteri* 180 efficiently transglucosylate Stevia component rebaudioside A, resulting in a superior taste. *Sci. Rep.* **2018**, *8* (1), 1516.
- (16) MacGregor, E. A.; Janeček, Š.; Svensson, B. Relationship of sequence and structure to specificity in the α -amylase family of enzymes. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2001**, *1546* (1), 1–20.
- (17) Meng, X.; Gangoiti, J.; Bai, Y.; Pijning, T.; Van Leeuwen, S. S.; Dijkhuizen, L. Structure–function relationships of family GH70 glucansucrase and 4,6- α -glucanotransferase enzymes, and their evolutionary relationships with family GH13 enzymes. *Cell. Mol. Life Sci.* **2016**, *73* (14), 2681–2706.
- (18) Vujičić-Žagar, A.; Pijning, T.; Kralj, S.; López, A. C.; Eeuwema, W.; Dijkhuizen, L.; Dijkstra, W. B. Crystal structure of a 117 kDa glucansucrase fragment provides insight into evolution and product specificity of GH70 enzymes. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (50), 21406–21411.
- (19) Koshland, D. E., Jr. Stereochemistry and the mechanism of enzymatic reaction. *Biol. Rev.* **1953**, *28* (4), 416–436.
- (20) Uitendaal, J. C. M.; Mosi, R.; Kalk, K. H.; van der Veen, A. B.; Dijkhuizen, L.; Withers, G. S.; Dijkstra, W. B. X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase elucidate catalysis in the α -amylase family. *Nat. Struct. Biol.* **1999**, *6* (5), 432–436.
- (21) Leemhuis, H.; Pijning, T.; Dobruchowska, J. M.; Dijkstra, B. W.; Dijkhuizen, L. Glycosidic bond specificity of glucansucrases: on the role of acceptor substrate binding residues. *Biocatal. Biotransform.* **2012**, *30* (3), 366–376.
- (22) Van Leeuwen, S. S.; Kralj, S.; Eeuwema, W.; Gerwig, G. J.; Dijkhuizen, L.; Kamerling, J. P. Structural characterization of bioengineered α -D-glucans produced by mutant glucansucrase GTF180 enzymes of *Lactobacillus reuteri* strain 180. *Biomacromolecules* **2009**, *10* (3), 580–588.
- (23) Kralj, S.; Eeuwema, W.; Eckhardt, T. H.; Dijkhuizen, L. Role of asparagine 1134 in glucosidic bond and transglycosylation specificity of reuteransucrase from *Lactobacillus reuteri* 121. *FEBS J.* **2006**, *273* (16), 3735–3742.
- (24) Irague, R.; Massou, S.; Moulis, C.; Saurel, O.; Milon, A.; Mosan, P.; Remaud-Simeon, M.; Portais, C. J.; Remaud-Simeon, G. NMR-based structural glycomics for high-throughput screening of carbohydrate-active enzyme specificity. *Anal. Chem.* **2011**, *83* (4), 1202–1206.
- (25) van Leeuwen, S. S.; Kralj, S.; van Geel-Schutten, I. H.; Gerwig, G. J.; Dijkhuizen, L.; Kamerling, J. P. Structural analysis of the α -D-glucan (EPS180) produced by the *Lactobacillus reuteri* strain 180 glucansucrase GTF180 enzyme. *Carbohydr. Res.* **2008**, *343* (7), 1237–1250.
- (26) Davies, G. J.; Wilson, K. S.; Henrissat, B. Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem. J.* **1997**, *321* (2), 557–559.
- (27) Pham, H. T. T.; Dijkhuizen, L.; van Leeuwen, S. S. Structural characterization of glucosylated lactose derivatives synthesized by the *Lactobacillus reuteri* GtfA and Gtf180 glucansucrase enzymes. *Carbohydr. Res.* **2017**, *449*, 59–64.
- (28) Bozonnet, S.; Dols-Laffargue, M.; Fabre, E. Molecular characterization of DSR-E, an α -1,2 linkage-synthesizing dextranase with two catalytic domains. *J. Bacteriol.* **2002**, *184* (20), 5753–5761.
- (29) Brison, Y.; Pijning, T.; Malbert, Y.; Fabre, E.; Mourey, L.; Morel, S.; Potocki-Véronèse, G.; Monsan, P.; Tranier, S.; Remaud-Siméon, M.; Dijkstra, B. W. Functional and structural characterization of α -(1 \rightarrow 2) branching sucrase derived from DSR-E glucansucrase. *J. Biol. Chem.* **2012**, *287* (11), 7915–7924.

- (30) Meng, X.; Pijning, T.; Dobruchowska, J. M.; Gerwig, G. J.; Dijkhuizen, L. Characterization of the functional roles of amino acid residues in acceptor-binding subsite + 1 in the active site of the glucansucrase GTF180 from *Lactobacillus reuteri* 180. *J. Biol. Chem.* **2015**, *290* (50), 30131–30141.
- (31) Meng, X.; Pijning, T.; Tietema, M.; Dobruchowska, M. J.; Yin, H.; Gerwig, G. J.; Kralj, S.; Dijkhuizen, L. Characterization of the glucansucrase GTF180 W106S mutant enzymes producing polysaccharides and oligosaccharides with altered linkage composition. *Food Chem.* **2017**, *217*, 81–90.
- (32) Van Geel-Schutten, G. H.; Faber, E. J.; Smit, E.; Bonting, K.; Smith, R. M.; Brink, T. B.; Kamerling, P. J.; Vliegthart, F. G. J.; Dijkhuizen, L. Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. *Appl. Environ. Microbiol.* **1999**, *65* (7), 3008–3014.
- (33) Nivedha, A. K.; Thieker, D. F.; Makeneni, S.; Hu, H.; Woods, R. J. Vina-Carb: improving glycosidic angles during carbohydrate docking. *J. Chem. Theory Comput.* **2016**, *12* (2), 892–901.
- (34) Trott, O.; Olson, A. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J. Comput. Chem.* **2010**, *31* (2), 455–461.
- (35) Meng, X.; Pijning, T.; Dobruchowska, J. M.; Gerwig, G. J.; Dijkhuizen, L. Characterization of the functional roles of amino acid residues in acceptor binding subsite + 1 in the active site of the glucansucrase GTF180 enzyme of *Lactobacillus reuteri* 180. *J. Biol. Chem.* **2015**, *290* (50), 30131–41.
- (36) van Leeuwen, S. S.; Leeflang, B. R.; Gerwig, G. J.; Kamerling, J. P. Development of a $(1)H$ NMR structural-reporter-group concept for the analysis of prebiotic galacto-oligosaccharides of the $[\beta\text{-d-Galp-}(1\rightarrow x)]n\text{-d-Glcp}$ type. *Carbohydr. Res.* **2008**, *343* (6), 1114–1119.
- (37) Meng, X.; Dobruchowska, J. M.; Pijning, T.; Gerwig, G. J.; Dijkhuizen, L. Synthesis of new hyperbranched α -glucans from sucrose by *Lactobacillus reuteri* 180 glucansucrase mutants. *J. Agric. Food Chem.* **2016**, *64* (2), 433–442.
- (38) Meng, X.; Pijning, T.; Dobruchowska, J. M.; Yin, H.; Gerwig, G. J.; Dijkhuizen, L. Structural determinants of alternating $(\alpha 1\rightarrow 4)$ and $(\alpha 1\rightarrow 6)$ linkage specificity in reuteransucrase of *Lactobacillus reuteri*. *Sci. Rep.* **2016**, *6*, 35261.
- (39) Meng, X.; Dobruchowska, J. M.; Pijning, T.; López, C. A.; Kamerling, J. P.; Dijkhuizen, L. Residue Leu940 has a crucial role in the linkage and reaction specificity of the glucansucrase GTF180 of the probiotic bacterium *Lactobacillus reuteri* 180. *J. Biol. Chem.* **2014**, *289* (47), 32773–32782.
- (40) Vuillemin, M.; Claverie, M.; Brison, Y.; Séverac, E.; Bondy, P.; Morel, S.; Monsan, P.; Moulis, C.; Remaud-Siméon, M. Characterization of the first α -(1 \rightarrow 3) branching sucrases of the GH70 family. *J. Biol. Chem.* **2016**, *291* (14), 7687–7702.
- (41) Pham, T. T. H.; Böger, C. L. M.; Dijkhuizen, L.; van Leeuwen, S. S. Stimulatory effects of novel glucosylated lactose derivatives GL34 on growth of selected gut bacteria. *Appl. Microbiol. Biotechnol.* **2018**, DOI: 10.1007/s00253-018-9473-8.